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E. Assous, C. Dreux, M. Girard

NEW APPLICATION OF DIALYSIS IN THE DETERMINATION OF AMMONIEMIA*

The problem of determination of NH_4^+ ions in the blood has long been studied by many authors. The techniques proposed, while they differ in the way in which they are used, are based on the same principle, one that can be summarized as follows. First, the NN_4^+ ions are displaced in a more or less strong alkaline medium in the form of gaseous ammonia, NH_3, which becomes fixed after diffusion in an acid solution, after which the NH_4^+ ions, thus isolated and collected, are evaluated by acidimetry or by colorimetry.

In a recent article** we looked at the methods employed most frequently at the present time and settled on the operative conditions under which these methods yield the best results.

However, the method used to separate the NH_4^+ ions can be subjected to certain criticisms. As a matter of fact:

the absolutely necessary alkaline medium can cause an NH_4^+ overcharge, upon hydrolysis of the nitrogenous ammoniogen conpounds, despite the precautions taken;

release of the gaseous ammonia, to be total, takes quite a long time, and this also can favor the spontaneous formation of supplementary NH_4^+ ions.

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* We thank Mrs. C. Dubois for her very valuable assistance.

** Girard, M., Assous, E., Canal, J., "Research to Find a Practical Determination of Ammoniemia," Ann. Biol. Clin., Vol. 17, No. 10-12, 1959, pp. 592-602.

This is why we asked ourselves if we could avoid the above inconveniences by finding another technique, one that would, on the one hand, have the advantage of avoiding the use of disturbing reagents, and on the other, provide for faster separation of the ammonium ions.

Only total, direct dialysis of the blood seemed to us likely to meet this double objective. This new application of dialysis was made possible by the use of the Technicon automatic analyzer.***

The latter, among other things, has the advantage of permitting a variety of successive operations (dialysis, the addition of coloring reagents, photometric reading) to be performed, in addition to being automatic.

The colorometric evaluation of the NH_4^+ ions was derived from the technique that uses Berthelot's sensitized phenolhypochlorite reaction, as we suggested in our earlier article.**

For purposes of convenience in reporting we prefer to outline first the techincal details of the method of operation adopted, then we will discuss its merits, particularly its selectivity and precision, and finally we will give the results for normal and pathological blood.

1. TECHNIQUE

It is not necessary to discribe here in detail the sequence of the operations performed automatically by the Technicon automatic analyzer, so we shall simply confine ourselves to setting down the composition of the reagents used, and the schematic diagram of the arrangement needed

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*** Automatic analyzer: Compagnie Technicon, 7 rue George-Ville (Paris, 16e).

to use our technique (fig. 1).

(A) REAGENTS:

1. Standard solution of ammonium ions NH_4^+:

(a) Mother solution 100 µg per ml.

Neutral ammonium sulfate, pure and dry. . . . . 0.3667 g

H_2SO_4 solution, about normal. . . . . 20 ml

Double distilled water, quantity sufficient for . . 1,000 ml

Or

Ammonuim chloride, pure and dry . . . . . 0.2972 g

H_2SO_4 solution about normal . . . . . 20 ml

Double distilled water, quantity sufficient for . . 1,00 ml

(b) Daughters solutions including respectively 0.25, 0.50, 1, 1.5, 2, 2.5, 3, 4, 5, µg of NH_4^+ ions/ml.

Shelf life excellent.

2. Sodium chloride solution:

Sodium chloride, pure and dry. . . . . 9 g

Double distilled water, quantity sufficient for. . 1,000 ml

Shelf life excellent.

3. Sodium phenol solution

Phenol, pure, crystallized. . . . . 25 g

Pure soda in pellets. . . . . 0.1 g

Double distilled water, quantity sufficient for . . 1,000 ml

pH about 8

Shelf life excellent in colored bottle.

4. Sodium nitroprusside solution

(a) Mother solution: Legal's reagent.

10% aqueous solution sodium nitroprusside . . . . . (in (equal

Glacial acetic acid . . . . . (parts

Shelf life excellent in colored bottle.

(b) Daughter solution obtained by diluting mother solution to 1/200.

Shelf life in colored bottle: one week.

5. Phosphate - sodium hypochlorite buffer solution:

Disoduim phosphate, Na_2HPO_4, 12 H_2O . . . . . 180 g

Pure soda in pellets. . . . . 6 g

Double distilled water, quantity sufficient for . . 1,000 ml

After dissolving add:

commercial sodium hypochlorite solution, at 55 degrees chlorometric, titrated by iodometry . . . . 10 ml

Shelf life excellent.

(B) Schematic diagram (Fig. 1):

Figure 1 shows the schematic diagram in accordance with the plan adopted for the determinations made using the Technicon automatic analyzer.

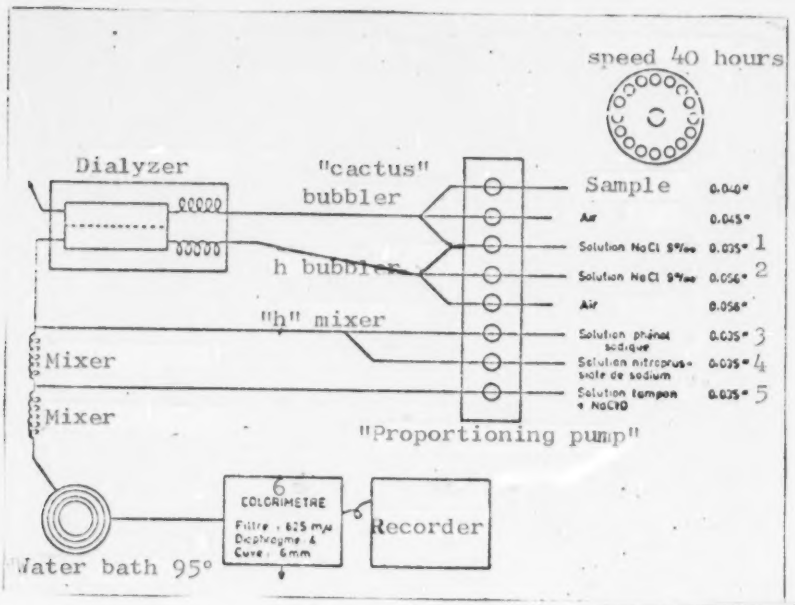

Figure 1.
Schematic diagram

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Key: 1 - NaCl Solution 9‰; 2 - NaCl Solution 9‰; 3 - Sodium phenol solution; 4 - Sodium nitroprusside solution; 5 - Buffer solution + NaClO; 6 - Colorimeter Filter 625 mμ Diaphragm 4 Cell: 6mm.

II. VALUE OF THE METHOD

In order to discuss the value of this method, it is necessary to verify the fact that the technique used will permit a selective determination of NH_4^+ ions to be made on the one hand, and to evaluate them as a whole, on the other.

(A) Selectivity of the method.

To determine this quality, it is essential to investigate in sequence:

1. the normal nitrogenous components of the blood not dialyzed, and which therefore cannot intervene in the subsequent colorimetric reaction of the dialyzed NH_4^+ ions;

2. the most favorable composition of the coloration reagents in

order that the derivatives accompanying the NH_4^+ ions in the dialyzate do not disturb the coloration furnished by the latter;

3. the reaction provided by the dialyzable nitrogenous substance in the absence of ammonium ions, given the experimental conditions outlined above. As a matter of fact, the method would lose all its value if, apart from the NH_4^+ ions, the other normal blood compounds would yield Berthelot's reaction.

1. Selectivity of the dialysis:

The dialysis membrane, the pores of which are 40 to 60 μ in diameter, retains the large protein and polypeptide molecules, but allows the small molecules, such as the crystalloids, urea, amino acids, and the like, to pass.

We studied the proportion in which the normal nitrogenous blood compounds capable of releasing NH_4^+ ions entered the dialyzated.

This percentage was evaluated by making determinations using the dialyzate and the residual fluid and the specific reactions of the different compounds studied in a pure solution.

Table I lists the results obtained with the NH_4^+ ions, urea, and the principal amino acids in the blood.

These results demonstrate that dialysis eleminates a high percentage of the principal nitrogenous derivatives capable of intervening during the subsequent coloration, in particular, glutamine.

2. Influence of reagent composition:

The only reagents that can be modified during the sensitized Berthelot coloration are the following:

the buffer solution, the pH of which can be varied to above the minimum value of 9.5 necessary for the appearance of a readable coloration.

the NaClO solution, the titer of which can be varied to above the minimum value of 0.10 degree chlorometric necessary for the linear progression of the coloration.

There are two groups of nitrogenous substances, determined previously, to be distinguished:

Table I

Substances	Results OD 10^{+3} on liquid		Percentage in the liquid	
	Dialyzed	Residual	Dialyzed	Residual
NH_4^+ ions	250	250	50	50
urea	210	210	50	50
Amino acids				
Alanine	5	260	2	98
Arginine	3	540	0,5	99,5
Aspartic(acid)	20	1620	1,2	98,8
Cystéine	5	310	1,6	98,4
Glutamine	2	540	0,4	99,6
Glutamic(acid)	5	350	1,5	98,5
Glycine	90	1300	6,5	93,5
Histidine	5	340	1,4	98,6
Lysine	5	260	1,9	98,1
Methionine	10	700	1,4	98,6
Oxyproline	50	210	19,2	80,8
Phenylalanine	1	48	2	98
Proline	7	55	11,3	88,7
Taurine	0,01	5	0,2	99,8
Thréonine	0,5	30	1,6	98,4
Valine	0,02	10	0,2	98,8

(a) substances that release NH_4^+ ions as a function of the pH of the hypochlorite concentration, (urea, glutamic acid, arginine, for example).

The following curves show graphically the importance of this release.

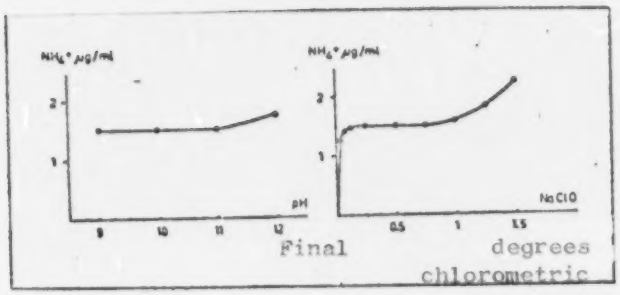


Figure 2.

After a plateau, seen in both cases, the release of NH_4^+ becomes accentuated beginning at pH 11 and at an NaClO concentration equal to 0.75 degree chlorometric;

(b) substances that release NH_4^+ ions as a function of the NaClO concentration up to a pH 10.5, but consume the hypochlorite at a higher pH in a manner such that they inhibit coloration (glutamine, cysteine, histidine, for example).

This hypochlorite consumption is placed in perspective by the following curves

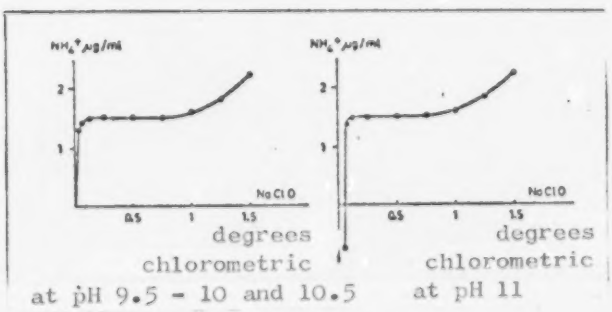


Figure 3.

Thus:

1. the pH must be higher than 9.5 in order to obtain a rather deep coloration, and lower than 10.5 to avoid NaClO consumption and the formation of an overcharge of NH_4^+ ions;

2. the NaClO concentration must be higher than 0.25 degree chlorometric for the coloration to evolve linearly, and must remain lower than 0.75 degree chlorometric in order for the NH_4^+ overcharge to be zero.

This is why we chose to fix the final pH at 10, and the hypochlorite concentration near 0.5 degree chlorometric.

3. Coloration selectivity applied to pure solutions of dialyzable nitrogenous compounds.

The optimum operative conditions having been determined once and for all, Berthelot's reaction was carried out on pure solutions of nitrogenous derivatives only slightly dialyzable, albeit still capable of disturbing the coloration. The following table (Table II) indicates the results of the coloration provided by urea, and by certain amino acids at concentrations higher than those in normal blood by a factor of 10.

The results indicate that the samples of the amino acids used, produce, although they are very weak, a positive Berthelot reaction, but at such low rates in relation to the quantity of the product used that it can be presumed that the coloration is due not to the substance used, but to ammoniacal impurities. It is impossible, however, to check this hypothesis directly because we could not find a more thorough purification than that performed on the samples used.

Table II

Substances	Concentration in µg/ml	NH_4^+ µg/ml	Ratio NH_4^+/substance
Urea	10 000	0,25	0,000 025
Amino acids			
Alanine	1 000	0,15	0,000 15
Arginine	1 000	0,2	0,000 2
Aspartic(Acid)	1 000	1	0,001
Cystéine	1 000	0,2	0,000 2
Glutamine	1 000	0,5	0,000 5
Glutamic(Acid)	1 000	0,2	0,000 2
Glycine	1 000	0,4	0,000 4
Histidine	1 000	0,05	0,000 05
Lysine	1 000	0,5	0,000 5
Méthionine	1 000	0,4	0,000 4
Oxyproline	1 000	0,1	0,000 1
Phénylalanine	1 000	0,02	0,000 02
Proline	1 000	0,1	0,000 1
Taurine	1 000	1	0,001
Thréonine	1 000	0,2	0,000 2
Valine	1 000	0,2	0,000 2

This is why we sought to use experiments that would be indirect proof of the preceeding hypotheses, the reasoning being as follows.

Let us determine, for each compound, the percentage of substance dialyzed by means of a specific colored reaction, involving successively the dialysis, and the residual, liquid. Then do the same experiment, but this time use Berthelot's reaction.

Two different results are thus obtained:

1. the ratio of dialyzed substance is the same in both cases (for example, 1/4 in Table III).

Given this possibility, it can be concluded that the product releases ammonium ions during the determination, or itself undergoes Berthelot's reaction;

2. the ratio of dialyzed substance is different in the two cases. If this ratio is very close to 1/2 in the Berthelot reaction, which is that obtained with the ammonium ions, it can be stated that Berthelot's reaction is not due to the substance under study, but to the ammoniacal impurities (Table IV).

Table III

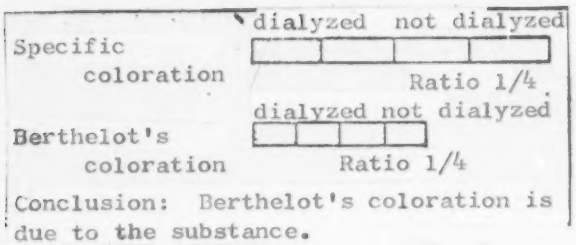
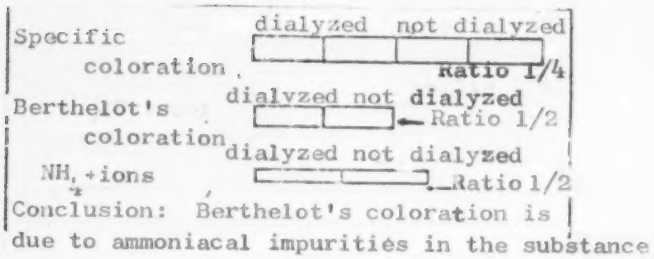


Table IV



We performed these experiments with a number of nitrogenous substances after setting up a system for determining the various products under study in the dialyzate, then in the residual liquid, using a specific color reaction (ninhydrin for the amino acids, diacetylmonoxime for the urea).

The results obtained are summarized in Table V.

Examination of the results shows that Berthelot's coloration, used on the dialysis liquid, and on the residual liquid of certain substances believed to be "ammoniogens", yields, as for the ammonium ions, a dialysis ratio of about 1/2.

It can, therefore, following our line of reasoning, be concluded that Berthelot's reaction is not caused by the substances presumed, but actually

by ammoniacal impurities, however slight, contained in the samples used.

(B) Accuracy of the method.

We have demonstrated that only NH_4^+ ions are capable of causing coloration, given the operative conditions defined.

It is still necessary to prove that all NH_4^+ ions are susceptible to evaluation in the same manner.

In fact, an overcharge of NH_4^+ ions added to a blood sample is completely recovered, as Table VI shows.

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Table V.

Substances	Specific coloration			Berthelot's coloration		
	1 Résultats en D.O. 10+3 sur liquide:		Ratio	NH_4^+ µg/ml		Ratio
	2 dialysé	3 résiduel	4 dialysé / total	5 dialysé	6 résiduel	7 dialysé / total
NH_4^+ ions				2	2	1/2
Urea	210	210	1/2	0,25	0,25	1/2
Amino acids						
Alanine	5	260	1/53	0,15	0,15	1/2
Arginine	3	540	1/181	0,2	0,2	1/2
Aspartic Acid	20	1620	1/82	1	1	1/2
Cystéine	5	310	1/63	0,2	0,2	1/2
Glutamine	2	540	1/271	0,5	0,5	1/2
Glutamic Acid	5	350	1/71	0,2	0,2	1/2
Glycocolle	90	1300	1/15	0,4	0,4	1/2
Histidine	5	340	1/69	0,05	0,05	1/2
Lysine	5	330	1/65	0,5	0,5	1/2
Méthionine	10	700	1/71	0,4	0,4	1/2
Oxyproline	30	210	1/5	0,1	0,1	1/2
Phénylalanine	1	48	1/49	0,02	0,02	1/2
Proline	7	55	1/9	0,1	0,1	1/2
Taurine	0,01	5	1/501	1	1	1/2
Thréonine	0,5	30	1/61	0,2	0,2	1/2
Valine	0,02	10	1/501	0,2	0,2	1/2

Key: 1 - OD Results in 10^{+3} on liquid; 2 - Dialyzed; 3 - Residual; 4 - Dialyzed; 5 - Dialyzed; 6 - Residual; 7 - Dialyzed.

Table VI.

Samples	NH_4^+ before overcharge µg/ml	Overcharge added µg/ml	NH_4^+ after overcharge µg/ml	Overcharge recovered µg/ml
N° 1	0,4	2	2,4	2
N° 2	0,7	2	2,7	2
N° 3	1,2	2	3,2	2
N° 4	2	2	4	2

(C) Conclusion:

The experiments conducted emphasize the specificity, and the precision, of the technique proposed.

The Technicon autoanalyzer adds the following advantages to these features: automation, simplicity, reproductibility, sensitiveness.

III. SOURCE AMMONIEMIA

We have tried to determine the conditions under which "source ammoniemia" can be evaluated, and, thus define the NH_4^+ ion level in the blood as soon as it leaves the vessels.

This study was made in two parts:

an experimental determination of source ammoniemia;

the temporal change in this level.

The results were such that conditions for blood sampling, and maximum shelf life for the blood samples, could be established.

(A) Experimental determination of source ammoniemia.

Determination is very difficult to establish by methods based on the diffusion of ammonium ions. In fact, the long period of time needed for complete diffusion, as well as the presence of alkaline reagents, create conditions capable of causing the formation of NH_4^+ ions from the ammoniogen compounds, with these ions being added to those already present in the blood at the time the blood is taken.

We returned to the problem with our method, which has the advantage of performing blood dialysis without the use of a reagent, and without pH modification. Another advantage of this technique is that it permits making successive determinations of the same blood sample every minute.

However, dialysis on the Technicon takes two minutes, and one also is led to believe that the values found do not correspond exactly to those of the source ammoniemia. However, the curve for ammoniemia variations plotted against time proves that the level does not vary for the first 45 minutes. It is, therefore, possible to believe, by extrapolation, that the NH_4^+ ion level determined two minutes after taking blood in fact represents the source ammoniemia.

Many determinations were made, using blood from controls. The mean of the values found in about one hundred determinations was 0.3 ± 0.2 mg of NH_4^+ ions per liter of blood.

The following table (Table VII) lists some of the values obtained for normal subjects, and, in some cases, for pathological subjects.

The normal values thus defined (0.3 ± 0.2 mg/liter) are shown to be definitely inferior to those obtained by diffusion methods (0.7 ± 0.3 mg/l).

The principle, as well as the methods used, are responsible for the differences noted in the definition of the respective normal values.

Table VII

Subjects	Diagnosis	Ammoniemia, mg/liter
DRE...	Control	0,3
ASS...	—	0,4
GIR...	—	0,2
DUB...	—	0,3
GIN...	—	0,3
MOR...	—	0,3
GOV...	—	0,5
GOM...	—	0,4
DAN...	—	0,2
ROS...	—	0,3
DEB...	Hepatic coma	2,40
JEN...	Hepatic coma	2,55
PAP...	Anastomosis porto-cave	1,8
PRA...	Cirrhosis	1,4
POS...	Cirrhosis	1

(B) Variation in source ammoniemia during storage of blood.

We then looked at the question of whether or not there is an increase in source ammoniemia when whole blood, or heparinized plasma, is stored. The purpose of this experiment was to fix the time limit beyond which the values found would no longer match those for source ammoniemia.

The following curves (Fig. 4) show the temporal variation in the ammoniemia level.

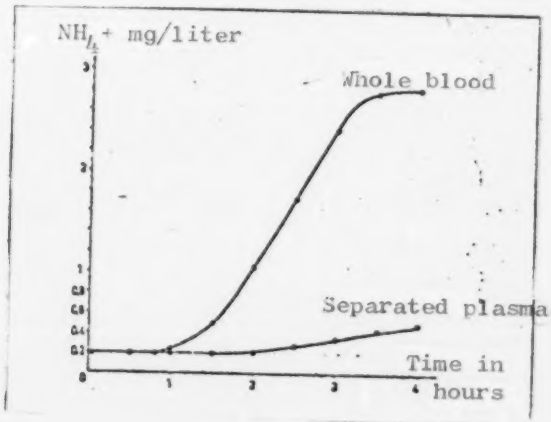

Figure 4.

It is to be noted that the temporal evolution of the ammoniemia level is different for plasma, separated immediately from corpuscles than for whole blood.

In the case of plasma, the values are unchanged for almost 90 minutes, with a definite increase appearing only about two hours later.

In the case of whole blood, on the other hand, evolution is much more rapid, with the level rising at the 45th minute, and reaching a value double that obtained at zero time after 85 minutes.

Thus, to obtain results identical with those of the source ammoniemia, it is necessary to follow exactly the following experimental conditions:

for plasma, an 80 minute wait is acceptable provided corpuscle separation is carried out within 45 minutes after the blood is drawn;

for whole blood, the determination must be made within 45 minutes after the blood is drawn.

Furthermore, it is advisable to use a drop of heparin, which has been checked beforehand for ammoniacal impurities, with the drawn blood.

CONCLUSION

The ammoniemia determination, made after blood dialysis yielded definitely inferior results to those obtained by diffusion methods, particularly by the technique we have recommended.** In fact, thanks to the advantages offered by the Technicon autoanalyzer it is possible to separate the NH_4^+ ions

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** See the article cited in the footnote on p. 1.

very rapidly, and without the contribution of alkaline reagents harmful to the specificity of the determination.

We thus have been able to determine a "source ammoniemia," defined as being that of extravasated blood as soon as the blood is drawn.

The experiment demonstrated that so far as established blood drawing and storage conditions are concerned, the value of the source ammoniemia remains constant long enough to make a precise determination.

Thus, the mean value of the source ammoniemia found in normal subjects is 0.3 ± 0.2 per liter of blood.

The proposed definition applies to NH_4^+ ions present in extravasated blood, but does not permit prejudging the existence of these ions in circulating blood.